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Overexpression of cyclin D1 and interaction between p27^{Kip1} and tumour thickness predict lymph node metastases occurrence in lower lip squamous cell carcinoma

Vito Rodolico [a,*], Federico Aragona [a], Daniela Cabibi [a], Cristofaro Di Bernardo [b], Roberto Di Lorenzo [b], Nicola Gebbia [c], Gaspare Gulotta [b], Vincenza Leonardi [b], Francesca Ajello [d]

[a] *Institute of Pathological Anatomy and Histology, University of Palermo, Via del Vespro 129, 90127 Palermo, Italy*
[b] *Department of General Surgery, Emergency and Organ Transplants (GENURTO), University of Palermo, Via L. Giuffrè 5, 90127 Palermo, Italy*
[c] *Department of Surgery and Oncology Disciplines, University of Palermo, Via L. Giuffrè 5, 90127 Palermo, Italy*
[d] *Department of Hygiene and Microbiology, University of Palermo, Via del Vespro 133, 90127 Palermo, Italy*

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Summary We have attempted to identify those subgroups of patients most likely to develop lymph node metastases from squamous cell carcinoma of the lower lip (LLSSC). A total of 97 subjects, who did not undergo elective neck dissection, were recruited into the 60-month disease-free survival study.

After univariate analysis, tumour size, histological grading, maximal thickness, perineural invasion and immunoreactivity to cyclin D1 and p27^{Kip1} proteins proved to be significant factors. Tests of the effect of interaction between p27^{Kip1} LI and tumour thickness yielded that the impact of tumour thickness on the risk of lymph node metastases was modified by the percentage of p27^{Kip1} positive cells. Subsequent to models of multivariate analysis, tumour size, positive cyclin D1 protein

* Corresponding author. Address: Via Rodolico, Via Principe di Belmonte n. 80, 90139 Palermo, Italy. Tel.: +39 91 6553538; fax: +39 91 6553521.
E-mail address: vitorodolico@virgilio.it (V. Rodolico).

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expression, maximal thickness (>5 mm), p27^{Kip1} LI (%) and the interaction term between p27^{Kip1} LI and tumour thickness retained strong independent predictive values for lymph node metastases. We suggest that immunohistochemistry for cyclin D1 and p27^{Kip1} may prove to be valuable ancillary tests for identifying LLSSC with metastatic potential.

Introduction

Lower lip squamous cell carcinoma (LLSCC) is a fairly common tumor found mostly in middle-aged or elderly males.[1,2] The prognosis for such patients depends mainly on the clinical staging of the neoplasia, especially with regard to its size and to lymph node status.[3–5]

A multivariate analysis of over 1000 patients with lip carcinoma confirmed that the subgroup having the lowest determinate survival of 61% at five-year involved subjects with regional metastases to the cervical lymph nodes[6]; these are observed at the time of initial lip carcinoma diagnosis in 2–12% of patients and another 3–13% will manifest nodal metastases sometime after the initial treatment for lip cancer.[2,6] For these reasons factors evidencing the potential aggressiveness of the tumour might be useful to identify which patients had a sufficient risk of occult lymph node metastases to justify elective regional treatment.

In recent years, numerous studies have attempted to identify new significant prognostic parameters and more suitable therapeutic approaches. Interesting results have been obtained with proteins directly and indirectly implicated in the regulation of the cell cycle.

Cyclin D1 (CCND1), located on chromosome 11q13, encodes a critical cell cycle regulatory protein implicated in cell cycle progression from G1 to S phase.[7] Both CCND1 amplification and overexpression have been reported to be significantly correlated to aggressive tumour growth and poor prognosis in many kinds of malignant tumours.[8–10]

p27^{Kip1} is a tumour suppressor gene located on chromosome 12p13. It encodes a nuclear protein which is a cdk inhibitor and inhibits the formation of cyclin D1/cdk complexes during G0 and early G1 phases of the cell cycle.[11] This inhibits inactivation of pRb and prevents G1 to S phase transition. Findings in several studies indicate that low, or absent, p27^{Kip1} protein expression is associated with tumour progression and a poor prognosis in several types of cancer,[12–15] and with regard to LLSC, we ourselves previously reported that a low expression of p27^{Kip1} protein is significantly associated to node metastases and to high microscopic thickness.[16]

Overexpression of cyclin D1 and underexpression of p27^{Kip1} have been shown to predict lymph node metastases in several malignancies [8–10,17,18]; their role in predicting lymph node metastases in LLSCC, however, has still not been investigated. The aim of the present study was to ascertain whether the cyclin D1 and p27^{Kip1} expression status, analysed by immunohistochemistry, might also predict the occurrence of lymph node metastases in surgically treated LLSCC patients, independently of other known predictive factors.

Materials and methods

Clinical and pathologic findings

We considered a group of 122 consecutive patients who underwent radical surgical resection of primary LLSCC between January 1st 1988 and December 31st 1998; tissue samples taken from the subjects and stored in the archives of the Institute of Pathologic Anatomy and Histology of the University of Palermo provided adequate histological material. Complete excision of the primary mass was ascertained through histological examination of the resected margins. The patients were followed up for at least five years after surgery, at 4-monthly intervals for the first year, 6-monthly intervals for the second and third years and annually thereafter. Nine patients were excluded because of histologically confirmed node metastases at the time of primary surgery. Sixteen patients were excluded because of insufficient follow-up. A total of 97 subjects (94 men and three women), who did not undergo elective neck dissection after palpation, ultrasound or computed tomography had shown no signs of any lymph node metastases, were recruited into the 60-month disease-free survival study.

Tumour size was measured in mm and then classified as T_1, T_2 and T_3, according to the TNM system.[19]

Histology

All the tissue samples were fixed in 10% buffered formalin, dehydrated in ethanol and

paraffin-embedded according to the routine technique. Sections 5 μm thick were cut and stained with H&E, PAS, Alcian blue and Masson's trichrome. They were reassessed with regard to histological grading and classified as G1, G2, G3 and G4, following the method recommended by Anneroth and Hansen.[20] Inspection for the possible presence of perineural invasion, reported as present or absent, was performed on each sample. The measurement of microscopic thickness was performed with a 2.5 magnifying lens by an image analysis system (Leica QM500, Cambridge, UK) and the resulting software processing was expressed in mm as previously described.[16]

Immunohistochemistry

The avidin–biotin peroxidase complex technique was used on 5 μm sections of formalin-fixed, paraffin-embedded tissues after deparaffinization as previously described.[16] Briefly, the sections were reacted consecutively with monoclonal antibody K25020 (Transduction laboratories, Lexington, KY, USA) generated from mouse $p27^{(Kip1)}$ protein at a concentration of 1:1.200 or, to detect cyclin D1, with mouse monoclonal antibody DCS-6 (DAKO, Glostrup, Denmark) at a dilution of 1:50 at 4°C overnight.

Biotinylated alpha mouse IgG obtained from horse serum (ABC Kit, Vector Laboratories, Burlingame, CA) was applied as a secondary antibody at 1:400 in PBS for 30 min at room temperature. Immunostaining was performed using the avidin–biotin peroxidase complex technique (ABC Kit, Vector Laboratories) applied for 30 min. Finally, 3,3-diaminobenzidine tetrahydrochloride (DAKO, Glostrup, Denmark) in distilled water was used as the chromogen for 10 min, and sections were counterstained using Mayer's hematoxylin.

The grade of $p27^{Kip1}$ protein expression in each specimen was evaluated according to the percentage of positively stained cells among the total number of counted cancer cells; all positive cells were counted regardless of intensity of staining and used as labeling index (LI). Cyclin D1 expression was assessed on both the intensity of nuclear staining within the tumour cells and the percentage of cells that were positive. Cytoplasmic staining was not regarded as positive expression. In general, tumours that showed strong nuclear cyclin D1 expression also showed diffuse distribution of positive cells within the tumour. These tumours were regarded as positive expressers of cyclin D1. Tumours that were negative expressers of cyclin D1 generally showed no cyclin D1 immunoreactivity at all. Tumours were divided into two groups, namely expressers of cyclin D1 and non-expressers of cyclin D1.

Statistical methods

Age of patients, tumour size, maximal thickness, histological grading, perineural invasion, cyclin D1 expression and $p27^{Kip1}$ LI were investigated as predictive factors for lymph node metastases. Since there were only three women patients in the group, the effect of gender was not investigated. The 5-year (60 months) disease-free survival rates were calculated using the Kaplan–Meier method and log-rank test.

Univariate and multivariate Cox proportional hazard regression models were used to estimate the effect of assumed predictive factors on the hazard of lymph node metastases within 60 months after primary surgery and 95% confidence interval (95% CI). To find a parsimonious model that adequately fits the data, backward stepwise procedure was used setting the significance criteria at $P = 0.05$ for variable inclusion in the final multivariate model and $P = 0.1$ for removal. Continuous variables were entered into Cox models either as such or as categorical parameter. Age of patients was categorised as <60 years, 60–70 years, >70 years; because there were very few tumours in class T_3, we chose to dichotomise size by pooling classes T_2 and T_3; histologic grading was dichotomised as G1 and G2 vs G3 and G4; tumour thickness was dichotomised on the basis of the third quartile (5 mm); cut-off point for dichotomising the percentage of neoplastic cells expressing $p27^{Kip1}$ was arbitrarily set at 20%.

To evaluate whether the risk of lymph node metastases was modified by interactions between the effect of cyclin D1 expression, $p27^{Kip1}$ LI, tumour size, maximal thickness, histological grading, and perineural invasion, the first level interaction term between pairs of variables was entered into separate multivariate Cox models.

The proportional hazards assumption was tested and proved not to be violated. Statistical two-tailed significance was set at $P = 0.05$. All analyses were performed with SPSS software (release 8.0; Chicago, IL, USA, 1997).

Results

The age of the 97 study patients, 3.1% female, ranged from 47 years to 88 years. (mean 67.3 years). Table 1 summarises the clinicopathological

features of patients at the beginning of follow-up. The depth of neoplastic invasion ranged from 1.16 to 17.45 mm (mean 4.73 mm ± 3.14 standard deviation); tumour size ranged from 3 mm to 51 mm (mean 16.05 mm ± 11.07 standard deviation). The median percentage of tumour cells with $p27^{Kip1}$ protein expression was 26.47% (range 0—94.50%).

Thirteen (13.40%) of the 97 patients subjected to follow-up developed late lymph node metastases. The overall 5-year (60 months) disease-free survival rate was therefore 86.60%.

Univariate analysis

Univariate Cox regression modelling (continuous variables as such) showed all the variables investigated, except patients' age, to be significant predictive factors for lymph node metastases risk. Similar results were obtained by testing continuous variables converted to categories (Table 2).

Multivariate analysis

Tests of the effect of interaction between pairs of variables on the hazard of lymph node metastases yielded significant results only for $p27^{Kip1}$ LI by tumour thickness as continuous ($P = 0.002$) as well as categorised ($P = 0.046$) variables. The effect of the interaction between $p27^{Kip1}$ LI and tumour thickness on mean time of disease-free survival is shown in Fig. 1. Patients with a tumour thickness

Table 1 Summary of clinicopathologic parameters in 97 lower lip squamous cell carcinomas

Parameter	No. of patients (%)
Sex	
Male	94 (96.9)
Female	3 (3.1)
Age (year)	
<60	18 (18.6)
60—70	20 (20.6)
>70	59 (60.8)
Size	
T_1	80 (82.5)
T_2	11 (11.3)
T_3	6 (6.2)
Histologic grade	
G1	11 (11.3)
G2	50 (51.6)
G3	27 (27.8)
G4	9 (9.3)
Maximal thickness	
⩽5 mm	73 (75.3)
>5 mm	24 (24.7)
Perineural invasion	
Absent	93 (95.9)
Present	4 (4.1)
Cyclin D1 protein expression	
Positive	65 (67.0)
Negative	32 (33.0)
$p27^{Kip1}$ protein expression	
LI ⩾ 20%	77 (79.4)
LI < 20%	20 (20.6)

Table 2 Univariate Cox regression models for predictors of lymph node metastasis with converted to categories and continuous variables

Categorised variables	Estimated hazard ratio	95% CI		P^a	Continuous Variables	Estimated hazard ratio	95% CI		P^a
		Lower	Upper				Lower	Upper	
Tumor size (T_{2-3} vs T_1)	15.21	2.25	94.18	0.0033	Tumor size (mm)	1.09	1.05	1.13	<0.0001
Maximal thickness (>5 mm vs ⩽5 mm)	13.17	3.61	48.02	0.0001	Maximal thickness (mm)	1.32	1.18	1.48	<0.0001
$p27^{Kip1}$ LI (LI < 20% vs LI ⩾ 20%)	9.37	0.02	0.27	<0.0001	$p27^{Kip1}$ LI (%)	0.92	0.89	0.96	<0.0001
Perineural invasion (present vs absent)	9.78	2.64	36.27	0.0006					
Cyclin D1 expression (positive vs negative)	7.94	2.18	28.90	0.0017					
Histologic grading (G3—4 vs G1—2)	3.51	1.15	10.75	0.028					

[a] Wald test.

>5 mm and p27^{Kip1} LI < 20% have a disease-free survival (37 months, 95% CI:24-49) significantly ($P = 0.039$) lower than patients whose tumour thickness is ⩽5 mm and p27^{Kip1} LI ⩾ 20% (59 months, 95% CI:58-61).

The backward stepwise procedure applied to a Cox proportional hazards regression model, initially including statistically significant continuous variables as such in univariate analyses and the interaction term between p27^{Kip1} LI and tumour thickness, identified only tumour size, positive Cyclin D1 protein expression, percentage of p27^{Kip1} positive tumour cells and the interaction term between p27^{Kip1} LI and tumour thickness as significant independent predictors of lymph node metastases (Table 3, model no. 1). The Wald test results on tumour thickness was not significant ($P = 0.072$); the likelihood ratio test, however, showed that when tumour thickness was removed a significant decrease in fit occurred; i.e. tumour thickness was needed in the model. A lower percentage of p27^{Kip1} LI, a larger grade of tumour thickness and size together with the expression of cyclin D1 in tumour cells, led to a higher risk of lymph node metastases.

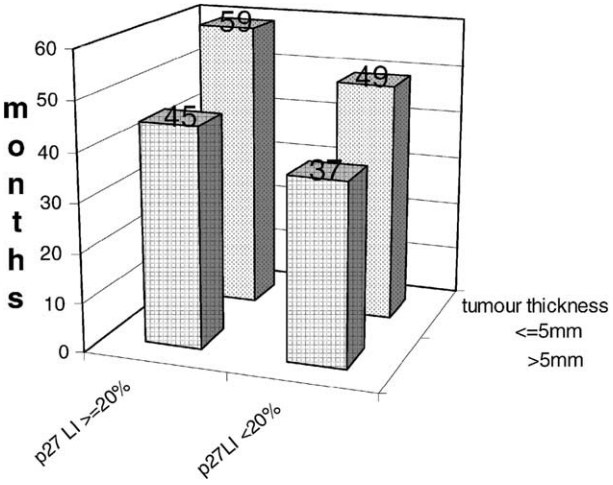

Figure 1 Mean time of metastatic events according to percentage of p27^{Kip1} positive tumoural cells and tumour thickness for 97 lower lip squamous cell carcinoma patients.

Backward stepwise multivariate Cox regression analysis was also carried out using the six categorical and categorised continuous variables statistically significant in univariate analyses (Table 3, model no. 2). Once more tumour size [T_2 and T_3 vs T_1: HR = 13.50 (95% CI: 2.19—83.06; $P = 0.005$)] and cyclin D1 [present vs absent: HR = 4.83 (95% CI: 0.99—23.37; $P = 0.0195$)] were retained in the final model that now included tumour thickness [>5 mm vs ⩽5 mm: HR = 10.93 (95% CI: 2.11—56.44; $P = 0.0043$)] as significant independent predictors of lymph node metastases. The p27^{Kip1} LI result of the Wald test, in fact, was not significant ($P = 0.069$); as for tumour thickness in the previous Cox model, however, p27^{Kip1} LI was also needed in this model.

Discussion

In this study, we have attempted to identify those subgroups of patients most likely to develop lymph node metastases from squamous cell carcinoma of the lower lip, to identify which patients

Table 3 Multivariate Cox proportional hazards regression model for predictors of lymph node metastasis: models with continuous (model no. 1) and converted to categories (model no. 2) variables

	Estimated hazard ratio	95% CI		P^a
		Lower	Upper	
Model no. 1				
Tumour size (mm)	1.04	0.99	1.09	0.04236
Cyclin D1 expression (positive vs negative)	13.13	1.77	91.89	0.0195
p27^{Kip1} LI (%)	0.70	0.57	0.88	0.0019
p27^{Kip1} LI (%) by maximal thickness (mm)	1.03	1.01	1.06	0.0053
Maximal thickness (mm)	0.96	0.74	1.25	0.072
Model no. 2				
Tumor size (T_{2-3} vs T_1)	13.50	2.19	83.06	0.005
Cyclin D1 expression (positive vs negative)	4.83	0.99	23.37	0.0195
Maximal thickness (>5 mm vs ⩽5 mm)	10.93	2.11	56.44	0.0043
p27^{Kip1} LI (LI < 20% vs LI ⩾ 20%)	2.28	0.47	11.09	0.069

[a] Wald test.

had a sufficiently high risk of occult lymph node metastases to justify elective regional treatment.

In this assessment of lymph node metastases predictors, we included five clinical and histopathological parameters (patient age, tumour size, histological grading, maximal thickness, perineural invasion) reported to be important predictors for survival in LLSSC; we hypothesised that the same factors might also predict the metastatic potential of such tumours. Finally, we included tumour immunoreactivity to cyclin D1 and p27^{Kip1} proteins based on the findings in other cancers.

After univariate analysis all the variables investigated, except patients' age, proved to be significant factors for lymph node metastases risk. Tests of the effect of interaction between p27^{Kip1} LI and tumour thickness yielded that the impact of tumour thickness on the risk of lymph node metastases was modified by the percentage of p27^{Kip1} positive cells. Subsequent to both models of multivariate analysis, including continuous and categorical variables, histological grading and perineural invasion lost their significance as predictors of lymph node metastases. However, tumour size, positive cyclin D1 protein expression, maximal thickness (>5 mm), p27^{Kip1} LI (%) and the interaction term between p27^{Kip1} LI and tumour thickness retained strong independent predictive values for lymph node metastases.

Studies in oral cancer at various subsites and stages have suggested that tumour thickness was a predictor of nodal metastasis and poor outcome,[21–23] although contrasting results have been reported.[24] Recent studies regarding the carcinoma of the tongue have demonstrated that tumour thickness was the powerful predictive factor for postoperative cervical nodal metastasis and disease-free survival.[22] There are only a few studies concerning tumour thickness in LLSCC. Onercl et al. found a cut-off at a tumour thickness of 5 mm above which the cervical lymph node metastasis rate was significantly increased.[25] Frierson and Cooper, using a cut-off of 6 mm of invasion, identified lymph node metastases in 75% compared to 4% of tumours measuring less than 6 mm.[4] In the present study we show that a tumours' thickness >5 mm has a significant independent predictive value for the occurrence of postoperative lymph node metastases in LLSCC.

We obtained intriguing results with regard to interaction between p27^{Kip1} LI and tumour thickness on the hazard of lymph node metastases; the data observed suggest that the diminution of the percentage of p27^{Kip1} positive tumour cells is a more powerful predictor of node metastases in patients who had greater tumour thickness than in those of small thickness. In other words, the effect of thickness on the hazard of lymph node metastases depends on the percentage of p27^{Kip1} positive tumour cells. The discrepancy observed between the two multivariate Cox models might depend on the loss of information occurring at times when continuous variables are converted into categories. Though absent or low expression of p27^{Kip1} protein has been shown to indicate poor prognosis for many tumours, the precise biological role of p27^{Kip1} in human tumours is still unclear. We previously reported an inverse correlation between p27^{Kip1} protein expression and microscopic thickness in LLSSC.[16] In the present study, we found that a low percentage of p27^{Kip1} positive tumour cells also represents a significant independent predictor of lymph node metastases occurrence.

It has been shown that patients with colorectal,[17] breast,[14] prostate,[15] or non-small cell lung cancer[12] with low or absent p27^{Kip1} protein expression had a poor prognosis. It has been reported that p27^{Kip1} expression had independent prognostic value in primary human colorectal carcinomas and that carcinomas with low or absent p27^{Kip1} protein displayed enhanced proteolytic activity specific for p27^{Kip1}[26]; this suggested that low p27^{Kip1} expression may result from increased proteosome mediated degradation rather than altered gene expression. Several studies have suggested that p27^{Kip1} may have additional functions such as cell–cell adhesion.[27,28] Thomas et al. have observed a reduction of p27^{Kip1} expression in metastasis of colorectal carcinoma compared to primitive tumours.[17] These findings may indicate that loss of p27^{Kip1} expression confers to tumour epithelial cells the ability to survive without anchorage and provide the tumour cells the opportunity to invade any tissue and to develop metastases.

As to cyclin D1 expression in LSCC, Fabbrocini et al. reported a 45.7% protein positivity incidence in a series of LLSCC compared to 0% of controls.[29] In own our series of LLSCC we observed a 67% cyclin D1 protein positivity; moreover multivariate analysis identified a significant independent predictive value for lymph node metastases occurrence.

Although the contribution of CCND1 gene amplification to overexpression on aggressive malignant phenotype has been well documented,[8] other likely mechanisms include up-regulation of receptor and signalling pathways that converge on cyclin D1 gene expression.[30] Recent evidence that levels of TGF and EGFR are independent predictors of outcome in patients with head and neck squamous cell carcinomas (HNSCC) raises the interesting possibility that part of this effect may be mediated via increased expression of cyclin D1.[31] Overexpression

of cyclin D1 shortens the G1 phase and reduces dependence on growth factors, which in turn may result in loss of cell cycle control and increased cell proliferation. However, in vivo infusion of iododeoxyuridine used as a measure of cell proliferation in a series of HNSCC, showed no correlation between cyclin D1 expression and cell proliferation.[32]

In conclusion, the present study identifies tumour size, maximal thickness, underexpression of $p27^{Kip1}$, overexpression of cyclin D1 and interaction between $p27^{Kip1}$ LI and tumour thickness as significant independent predictors of lymph node metastases in LLSSC. Although tumour size and maximal thickness have previously been identified as important factors associated with lymph node metastases, this is the first report linking the interaction between $p27^{Kip1}$ protein and tumour thickness, the overexpression of cyclin D1 and the underexpression of $p27^{Kip1}$ to lymph node metastases occurrence in LLSSC. Immunohistochemistry for cyclin D1 and $p27^{Kip1}$ can be used in routine diagnosis and even on biopsy material prior to surgery and is suitable for use in the selection of a high-risk subgroup of patients who might benefit from a more aggressive therapeutic approach; these variables may prove to be valuable ancillary tests for identifying LLSSC with metastatic potential.

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